mTOR-Maf1 signaling pathway regulates dendritic development induced by Reelin

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Running title: Reelin induces Pol III transcription via Maf1

SUMMARY

Reelin plays a fundamental role in brain development by stimulating signaling pathways including the one mediated by mTOR. However, the downstream event of mTOR signaling activated by Reelin remains largely unknown. Here, we report that Reelin promotes Maf1 phosphorylation, nucleus exclusion of Maf1, and elevated RNA Polymerase III (Pol III) transcription in mouse primary neurons. We showed that Maf1 is regulated by Reelin and mTOR. In addition, we demonstrated that PI3K-mTOR signaling is required for Reelin induced transcription in neurons. We further showed that Reelin promotes mTOR enrichment at the promoters of Pol III-transcribed genes. Remarkably, short-hairpin RNA knockdown of Maf1 stimulates a robust dendritic development by significant enhancement of Pol III transcription, while over-expression of Maf1 abolishes such effect of Reelin. Thus, we identified a novel role of Maf1 in neurons and established a molecular mechanism of Reelin-induced dendrite outgrowth and spine development.

INTRODUCTION

Reelin, the extracellular matrix glycoprotein, plays crucial roles in embryonic and adult brains. During early cortical development, Reelin controls proper migration and positioning of cortical neurons, while in late developmental stage, it plays a role in promoting extension of dendritic processes and development of dendritic spines (D'Arcangelo et al., 1995; Niu et al., 2004; Niu et al., 2008). In the adult cortex, Reelin is required for modulating synaptic plasticity (Beffert et al., 2005; Weeber et al., 2002). Reelin deficiency is associated with neurological disorders such as schizophrenia, autism and Alzheimer's disease (Fatemi, 2001; Herring et al., 2012). Although the upstream signaling components activated by Reelin are well-characterized, the downstream Reelin signaling event remains poorly defined. Recent findings have revealed that the mechanistic target of rapamycin (mTOR) signaling is activated by Reelin (Jossin and Goffinet, 2007; Ventruti et al., 2011). However, the mechanism of Reelin-regulated neuronal functions such as dendritogenesis at molecular level remained to be determined.

Emerging evidence has shown that mTOR plays a role in regulation of various aspects of neuronal functions including neuronal growth, development, synaptic plasticity and repair (Don et al., 2012; Jaworski and Sheng, 2006; Swiech et al., 2008). Current understanding of neuronal mTOR functions is mainly on its central role in controlling mRNA translation locally within axons for generation of new growth cones and axon elongation. mTOR-mediated protein synthesis has also been implicated in dendrite development (Jung et al., 2012). Recent studies in our laboratory and others have shown that mTOR plays important role beyond regional mRNA translation. We first showed in exponentially growing yeast that TOR is associated with ribosomal RNA (rRNA) and transfer RNA (tRNA) gene promoters, which are critical for their transcription and cell growth (Li et al., 2006; Wei et al., 2009). We and others subsequently demonstrated that mTOR in proliferating mammalian cells also binds to the promoters of these genes, suggesting that mTOR plays a conserved role in transcription regulation for cell growth (Kantidakis et al., 2010; Shor et al., 2010; Tsang et al., 2010). However, the role of mTOR-

mediated transcription in non-dividing cells such as neurons is yet to be determined. In this study, we explored the transcription regulatory role in neurons and discovered that mTOR signaling controls transcription in neuronal cell lines and primary neurons. In addition, we demonstrated that such mTOR-regulated transcription is activated by Reelin, which plays a critical role in dendritogenesis in cortical and hippocampal neurons. Furthermore, we identified that Maf1, a conserved mTOR substrate, plays a crucial role in regulation of dendritic growth and spine formation. Therefore, we present a novel role of Maf1 in neuronal development and define a molecular mechanism by which Reelin controls dendritogenesis through transcriptional regulation mediated by mTOR signaling pathway.

RESULTS

mTORC1-Maf1 signaling is required for Pol III-dependent transcription in primary neurons

We have previously shown that TOR complex 1 (TORC1) plays a critical role in regulation of transcription by RNA polymerase III (Pol III) in yeast and proliferating mammalian cells (Li et al., 2006; Tsang et al., 2010; Tsang and Zheng, 2007; Wei et al., 2009). To explore the transcription regulatory function of mTORC1 in neuronal cells, we inhibited mTORC1 by rapamycin in primary embryonic cortical neurons, and determined transcription of Pol III-dependent genes. As shown in Figure 1A, mTORC1 is completely inhibited by rapamycin as revealed by the dephosphorylation of mTOR and its downstream substrates S6K1 and 4EBP1. To determine Pol III-dependent transcription, we measured the transcription level of 5S rRNA and $tRNA^{LEU}$ genes, which are the major Pol III targets. By RT-PCR, we measured the levels of 5S precursor rRNA (pre-rRNA) and pre-$tRNA^{LEU}$ transcripts, which are rapidly processed after transcription and therefore their levels can accurately reflect the transcription activity. As shown in Figure 1B, rapamycin dramatically reduced 5S rRNA and $tRNA^{LEU}$ gene transcription. Such effect of

rapamycin was also observed in hippocampal neurons and N2A cells (Figure S4A, S1B). To test whether mTORC2 plays a role in Pol III-transcription, we treated primary cortical neurons with Pp242, an ATP-competitive kinase inhibitor that inhibits both mTORC1 and mTORC2 activity. Treatment of Pp242 did not further reduce Pol III-dependent transcription (Figure 1C), suggesting that mTORC1, but not mTORC2, plays a major role in Pol III-dependent transcription. To directly demonstrate that mTORC1 regulates Pol III transcription, we knocked down the essential mTORC1 subunit Raptor (Figure 1D) by shRNA. Consistent with the rapamycin effect, depletion of Raptor by shRNA also caused reduction of 5S pre-rRNA transcript level (Figure 1E). These results demonstrate that mTORC1 (for simplicity, we use mTOR as mTORC1 hereafter) regulates Pol III-dependent transcription in neuronal cells.

We previously demonstrated that DNA binding activity of mTOR is required for promoting transcription (Li et al., 2006; Tsang et al., 2010). To further support the role of mTOR in transcriptional regulation in primary neurons, we performed ChIP analysis of mTOR in the absence or presence or rapamycin. In primary cortical neurons, we showed that rapamycin caused dissociation of mTOR from the promoters of 5S rRNA and $tRNA^{LEU}$ genes (Figure 1F). Similar result was also observed in Neuro2A cells (Figure S1A). These results further support a role of mTOR in regulation of 5S rRNA and tRNA gene transcription. To gain a mechanistic insight of mTOR-mediated Pol III-dependent transcription, we examined Maf1 because it is a key transcriptional repressor of Pol III transcription (Boguta, 2012; Geiduschek and Kassavetis, 2006; Willis and Moir, 2007). Moreover, we and others have previously found that Maf1 is a novel downstream target of TOR in yeast and mammalian cells (Michels et al., 2010; Wei et al., 2009). Thus, we investigated the role of Maf1 in mTOR-regulated transcription in neurons. Maf1 is a phosphoprotein and it migrates as a doublet on SDS-PAGE, with hypophosphorylated protein being the faster migrating form and active repressor of Pol III transcription, whereas the slow migrating form is hyper-phosphorylated protein which is inactive in transcription repression (Goodfellow et al., 2008; Wei et al., 2009). We first examined whether Maf1 is regulated by

mTOR in neuronal cells, we expressed Myc-tagged Maf1 proteins in Neuro2A cells and measured Maf1 phosphorylation by electrophoretic mobility shift. In the absence of rapamycin, Maf1 existed as phosphorylated (upper band) and dephosphorylated (lower band) forms, while rapamycin caused Maf1 dephosphorylation (Figure 1G). We also confirmed this observation in endogenous Maf1 protein in primary cortical neuron (data not shown). Typically, Maf1 is accumulated in the nucleus for repression of Pol III transcription after mTOR inhibition in yeast and mammalian cells (Shor et al., 2010; Wei et al., 2009). To confirm Maf1 is regulated by mTOR for its transcription regulation in primary neurons, we determined Maf1 subcellular localization by immunofluorescence imaging. In normal growth medium without rapamycin, Maf1 was mainly localized in the cytoplasm. In contrast, rapamycin treatment caused prominent Maf1 accumulation in the nucleus of primary hippocampal and cortical neurons (Figures 1H and S2). This observation suggests that dephosphorylation of Maf1 by rapamycin leads to its nuclear accumulation and active repression of Pol III transcription. Together, these results demonstrate that Pol III transcription is regulated by mTOR-Maf1 signaling in neurons.

Reelin induces Pol III-dependent transcription in primary neurons

To examine whether the physiological activation of mTOR signaling promotes Pol III-dependent transcription, we focused on Reelin because this extracellular glycoprotein can stimulate mTOR signaling pathway in neurons (Jossin and Goffinet, 2007; Ventruti et al., 2011). We confirmed that treatment of primary neuronal cultures with recombinant Reelin resulted in activation of mTOR pathway (Figure 2A). Intriguingly, we found that Reelin promoted Maf1 phosphorylation as the slow migrating form of Maf1 was increased after Reelin treatment (Figure 2B). Moreover, Reelin caused prominent redistribution of Maf1 in the cytoplasm of primary neurons (Figure 2C). We further confirmed this observation by performing in vivo study of Maf1 localization. We examined the neocortex of P0 mice because of the presence of Reelin in this region. We showed that Maf1 is mainly localized in the cytoplasm of neurons in different cortical layers of

wild type mice (Figure 2D). In contrast, Maf1 became more concentrated in the nucleus of the cortical neurons in *reeler* mouse (Figure 2D). To investigate the role of Reelin in activation of Pol III transcription, we determined expression of 5S rRNA and tRNA genes in response to Reelin treatment. As shown in Figure 2E, Reelin treatment significantly stimulated 5S pre-rRNA, $tRNA^{TYR}$ and $tRNA^{LEU}$ synthesis (Figure 2E, F). Moreover, we performed ChIP analysis to investigate the effect of Reelin on mTOR-DNA binding to Pol III transcribed gene promoters. As shown in Figure 2G, Reelin treatment led to increased binding of mTOR to the promoters of 5S rRNA and $tRNA^{LEU}$ genes. These results demonstrate that Reelin promotes Pol III-dependent transcription in neuronal cells. Importantly, these results suggest that Maf1 is a novel target of Reelin signaling pathway for regulation of its neuronal functions.

PI3K mediates Reelin-induced mTOR-Maf1 signaling for transcription regulation by Pol III

Our results showing that Reelin increased mTOR recruitment to the promoters (Figure 2G) and elevated transcription by Pol III (Figure 2E,F) suggest that mTOR plays a critical role in Reelin-induced Pol III transcription. To confirm this, we pre-treateded culture of primary cortical neurons with rapamycin and then incubated in Reelin conditioned medium in the presence of rapamycin and analyzed by western and RT-PCR. Pre-treatment of rapamycin blocked Reelin-induced mTOR signaling activation as revealed by S6K (T-389) phosphorylation (Figure 3A lane 2,3), and completely abolished Reelin-induced 5S pre-rRNA and tRNA gene transcription (Figure 3B-D). To further characterize the signaling pathway required for Reelin-induced Pol III-dependent transcription, we investigated the role PI3K because it is one of the major upstream regulators of mTORC1 (Tsang et al., 2007). We inhibited PI3K by pre-treating primary neurons with Ly294002 before incubation with Reelin conditioned medium. Ly294002 inhibited PI3K as revealed by AKT (T-308) dephosporylation (Figure 3A, lane 4), and completely blocked Reelin-induced 5S pre-rRNA transcription (Figure 3B lane 6, 3C). Similar results were also observed in $tRNA^{LEU}$ transcription (Figure 3D). We repeated the experiments in primary hippocampal

neurons and the results were consistent (Figure S3). These results reveal that Reelin induces Pol III-dependent transcription through activation of PI3K-mTOR signaling in primary neurons.

Maf1 is an intrinsic negative regulator of neurite outgrowth in primary neurons

The fact that Maf1 is a major transcription repressor of Pol III (Willis and Moir, 2007) prompted us to investigate whether ablation of Maf1 may enhance Pol III transcription in primary neurons. To test this hypothesis, we knockdown Maf1 and determined the transcription of 5S rRNA gene in primary neurons. As shown in Figure 4A, Maf1 was effectively knocked down by the shRNA. Indeed, Maf1 knockdown led to significant induction of 5S pre-rRNA (Figure 4B,C) and tRNA (Figure 4D). These results indicate that Maf1 is a negative regulator of Pol III-dependent transcription in primary neurons. To investigate the functional role of Maf1 in neuronal growth and development, we determined the effect of Maf1 ablation on the growth of neurite processing in primary hippocampal neurons. Primary hippocampal neurons transfected with Maf1 shRNA plasmid was determined by positive GFP signal, and their total neurite lengths were measured by immunofluorescence imaging using β-tubulin III staining. As shown in Figure 4E, knockdown of Maf1 led to robust increase in neurite outgrowth and branching compared with scramble control. In addition, the Maf1 knockdown-induced neurite outgrowth was not inhibited by rapamycin (Figure 4E,F). These results demonstrate that removal of the intrinsic repressor of Pol III can lead to robust stimulation of neurite outgrowth, and that Maf1 is the major mTOR substrate responsible for regulation of neurite outgrowth in primary neurons.

To confirm the role of Maf1, we over-expressed Maf1 by transfection of primary neurons with pCMV-Myc-Maf1 plasmid and compared the expression of Pol III-dependent genes with those transfected with control vector. We found that over-expression of Maf1 caused reduction of Pol III-dependent transcription (Fig. 4G,H). In addition, over-expression of Maf1 caused a moderate but significant reduction of total neurite length in primary hippocampal neurons

compared with cells transfected with control vector (Figure 4I,J). These results demonstrate that Maf1-mediated Pol III-dependent transcription plays a key role in neurite outgrowth.

Maf1 is a key regulator for Reelin-induced dendritic growth and spine development

One of the important functions of Reelin is to induce dendritic development and spine formation in hippocampal neurons during brain development (Niu et al., 2004; Niu et al., 2008). Our data showing that Reelin up-regulates Pol III-dependent transcription and that Maf1 is critical regulator for neurite outgrowth suggest that Reelin-induced dendritic growth is mediated by Maf1-regulated Pol III-transcription. To test this hypothesis, we incubated the primary hippocampal neurons transfected with control vector or plasmid over-expressing Maf1 in Mock or Reelin conditioned medium. As expected, Reelin notably induced neurite outgrowth compared with neurons incubated in mock medium (Figure 4K), which is in agreement with previous studies (Niu et al., 2004; Niu et al., 2008). Remarkably, over-expression of Maf1 substantially abolished Reelin-induced neurite outgrowth (Figure 4K), suggesting that Maf1-mediated Pol III-dependent transcription plays an important role in Reelin-induced neurite outgrowth in hippocampal neurons. As it is reported that Reelin promotes spine formation during dendrite development (Niu et al., 2008), we analyzed dendritic spines by immunofluorescnece imaging. Intriguingly, Maf1 knockdown induced robust spine formation along the dendrites of primary hippocampal neurons even in Mock medium (Figure 4L). Surprisingly, the effect of Maf1 knockdown on spine development was more robust than that induced by Reelin which was served as a positive control in this experiment (Figure 4L). To confirm the negative regulatory role of Maf1, we over-expressed Maf1 and incubated primary hippocampal neurons in Reelin conditioned medium. As shown in Figure 4M, Maf1 over-expression clearly reduced spine density and length induced by Reelin. Collectively, these results demonstrate that Maf1 is a novel neuronal transcriptional regulator that plays crucial role in controlling dendritic outgrowth and spine development induced by Reelin.

DISCUSSION

In this study, we identified a novel functional role of Maf1 in regulation of dendritogenesis in primary embryonic neurons. Moreover, we uncovered the mechanism by which Reelin controls dendritic development through PI3K-mTORC1-Maf1 signaling pathway by promoting Pol III-dependent transcription. Based on the present findings, we propose a model of Reelin-induced dendritic development (Figure 4N).

Although Pol III transcription is known to play important role in cell growth in proliferating cells (Goodfellow and White, 2007), its role in non-diving cells such as neurons is poorly defined. In this study, we discovered that Pol III-dependent transcription is critical for dendritogenesis in primary neurons. How does an increase in Pol III-dependent transcription drive dendritogenesis? Given that Pol III synthesizes 5S rRNA, tRNA and other untranslated RNA molecules that are essential components of the translation machineries, we propose that stimulation of Pol III transcription is critical for supporting the increased demand for protein synthesis and actin organization required for dendrite development. Importantly, our results highlight the critical importance of mTOR-mediated transcription for the production of protein synthesis machineries, in addition to the prevailing view that mTOR functions in promoting local mRNA translation in growing dendrites and axons (Jung et al., 2012). Therefore, both transcription and local translation regulated by mTOR signaling may be critical for dendritogenesis during embryonic development or neuronal regeneration after injury in adult brain. Although neurons are terminally differentiated cells, they show growth capability including the dendritic plasticity during embryonic development or neuronal regeneration at adult stage. Thus, the present study implies that Maf1 is a potentially important pharmacological target for treatment of

neurodegenerative diseases or neuronal regeneration after brain injuries. Future studies using animal models of traumatic brain injury or neurodegenerative diseases will shed important light on its therapeutic potential. Furthermore, Reelin expression is decreased in schizophrenia, autism, depression, temporal lobe epilepsy and Alzheimers disease, our findings that PI3K-mTOR-Maf1 signaling and Pol III-dependent transcription play critical role in Reelin-mediated function will open an avenue for research on studying the underlying molecular mechanisms and therapeutic intervention in these neurological and psychiatric disorders caused by Reelin insufficiency.

EXPERIMENTAL PROCEDURES

RNA isolation, RT-PCR and real time PCR

Total RNA was isolated and purified as described previously (Tsang et al., 2010). Reverse transcription was performed using random decamers and the RETROscript Kit (Ambion) according to the manufacturer's instruction. cDNA was then amplified by PCR as described in the manufacturer's instructions. For real time PCR, cDNA levels were analyzed using the Rotor-Gene Q 2plex System (Qiagen). To ensure the samples were free from DNA contamination, control samples in which the reverse transciptase was omitted during cDNA synthesis were run. Each sample was tested in triplicates using the Rotor-Gene SYBR Green PCR Kit as described in the manufacturer's manual. The thermocycling program consisted of one hold at 95 °C for 5 min, followed by 40 cycles of 5 s at 95 °C and 10 s at 60 °C. After completion of these cycles, melting-curve data were then collected to ensure PCR specificity, contamination and the absence of primer dimmers. mRNA levels of *GAPDH* or *ACT1* were used for normalization. Relative RNA levels were determined by standard curves method as described (Nolan et al., 2006). The following primers were used for conventional and real time PCR: 5S pre-rRNA,

GGCCATACCACCCTGAACGC (forward), CAGCACCCGGTATTCCCAGG (reverse); pre-tRNA^{LEU}, AGGATGGCCGAGTGGTCTAA (forward), TCCCCAGACAGGGAAGCTAA (reverse); pre-tRNA^{TYR}, CCTTCGATAGCTCAGCTGGT (forward), GTGGTAGTACACCCGTACTC (reverse); β Actin, GACGGCCAGGTCATCACTAT (forward), ACATCTGCTGGAAGGTGGAC (reverse); GADPH, GGTGAAGGTCGGTGTGAACG (forward), CTCGCTCCTGGAAGATGGTG (reverse). The primer pairs used to measure the 5S rRNA and tRNA transcription detected their nascent transcripts which are processed rapidly during transcription and therefore can accurately reflect their transcription activity.

SUPPLEMENTAL INFORMATION

Supplemental information includes four figures, Supplmental Experitmental Procedures, and Supplemental References are attached.

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FIGURE LEGENDS

Figure 1. mTORC1 regulates 5S rRNA and tRNA by RNA polymerase III in primary neurons. (A) Western blot analysis of the extracts prepared from primary cortical neurons for 3 days in vitro (DIV) treated with or without 20 nM rapamycin (Rap) for 4 hrs. (B) RT-PCR analysis of 5S precursor rRNA (pre-rRNA) and pre-tRNA^{LEU} in the extracts from (A). Reactions without DNA (No DNA) and without reverse transcriptase (No RT) were served as controls. Lower panel shows the quantification of transcriptional repression relative to the untreated controls. Results from three biological replicates are plotted ± SD. *, $p < 0.05$ comparing rapamycin treated sample with untreated control. (C) RT-PCR analysis of 5S pre-rRNA and pre-

$tRNA^{LEU}$ in the primary cortical neurons treated with DMSO, 20 nM rapamycin or 1 μM Pp242 for 4 hrs. (D) mTORC1 was genetically inactivated by shRNA against Raptor. Protein expression of Raptor was analyzed in primary cortical neurons for 3 DIV by western blot using an anti-Raptor antibody. (E) RT-PCR analysis of 5S pre-rRNA level in primary cortical neurons (3 DIV) transfected with scramble control or shRNA Raptor. Lower panel shows the quantification of relative 5S pre-rRNA level. (F) ChIP analysis of mTOR binding to the promoters of Pol III-transcribed genes in primary cortical neurons (3 DIV) treated without or with 20 nM rapamycin for 4 hrs. For control Ab, IgG was used instead of mTOR antibody. (G) Electrophoresis mobility shift analysis of Myc-Maf1 by western blotting. Neuro2A cells transfected with Myc-tagged Maf1 were treated with 20 nM rapamycin for 30 min and then subjected to western blot analysis using anti-Myc antibody to detect the mobility shift of Maf1. (H) Indirect immunofluorescence (IF) analysis of Maf1 localization in primary hippocampal neurons (5 DIV) treated with or without 20 nM rapamycin for 4 hrs. Localization of Maf1 was analyzed by anti-Maf1 antibody. Cell and neurite morphology were analyzed by beta-tubulin III staining and nucleus was stained by DAPI.

Figure 2. Reelin induces Pol III-dependent transcription in primary neurons. (A) Immunoblotting of extracts prepared from primary cortical neurons (3 DIV) incubated in Mock or Reelin conditioned medium for 4 hrs. (B) Electrophoresis mobility shift analysis of Maf1 by western blot. Primary cortical neurons (3 DIV) were incubated in Mock or Reelin conditioned medium for 30 min and then subjected to western blot analysis using anti-Maf1 antibody to detect the mobility shift. (C) IF analysis of Maf1 localization in primary hippocampal neurons. The primary neurons (5 DIV) were incubated in Mock or Reelin conditioned medium for 30 min and localization of Maf1 was analyzed by anti-Maf1 antibody. (D) Neocortical sections of postnatal P0 wild-type mice were processed for immunofluorescence analysis using anti-Maf1 antibody. The boxed image is enlarged for cellular distribution Maf1(green) and nucleus (red).

(E) RT-PCR analysis of 5S pre-rRNA in the extracts from (A). (F) RT-qPCR analysis of $tRNA^{TYR}$ and $tRNA^{LEU}$ in the extracts from (A). Data represent mean ± SD of expression level relative to Mock from triplicate experiments. *$p < 0.05$ (G) ChIP analysis of mTOR binding to promoters of 5S rRNA and $tRNA^{LEU}$ genes. Primary cortical neurons (3 DIV) were incubated in Mock (Mo) or Reelin (Ree) conditioned medium for 4 hrs and subjected to ChIP analysis.

Figure 3. Signaling characterization of Reelin-induced transcription regulation. (A) Immunoblotting of extracts prepared from primary cortical neurons of 3 DIV pre-treated with rapamycin or Ly294002 for 30 min, followed by incubation in Reelin conditioned medium containing the same concentration of drug for 20 min. Extracts were prepared and subjected to western blot analysis using anti-pS6K (T389) and anti-pAKT (T308) as the markers for mTOR and PI3K activity, respectively. (B) RT-PCR analysis of 5S pre-rRNA in the extracts from (A). (C) Quantification of result of (B). (D) RT-qPCR analysis of $tRNA^{LEU}$ in RNA extract from (A).

Figure 4. Maf1 mediates Reelin-induced Pol III transcription. (A) Maf1 was genetically depleted by Maf1 shRNA. Protein expression of Maf1 was analyzed by western blot using anti-Maf1 antibody in primary cortical neurons (3 DIV) transfected with scramble or Maf1 shRNA. (B) RT-PCR analysis of 5S pre-rRNA in the extracts from (A). (B) Quantification of result from B. (D) RT-qPCR analysis of $tRNA^{LEU}$ and $tRNA^{TYR}$ in RNA extract from (A). (E) IF analysis of neurites in primary hippocampal neurons transfected with Maf1 shRNA and treated in the absence or presence of rapamycin for 8 days. Transfected neurons were marked by GFP. Neurite morphology was visualized by beta-tubulin III antibody and nucleus was stained by DAPI. (F) Quantification of total neurite length in neurons in (E). Data are means ± S.D. of at least 50 randomly selected individual neurons per condition from two independent experiments. *, $p < 0.05$; No significant difference (s.d.), $p > 0.05$. (G) RT-PCR analysis of 5S pre-rRNA expression in primary cortical neurons (3 div) transfected with pCMV-Myc-Maf1 or control vector. (H)

Quantification of result from B. (I) IF analysis of neurites in primary hippocampal neurons transfected with pCMV-Myc-Maf1 plasmid or control vector for 8 days. Neurite morphology was visualized as in (E). (J) Quantification of total neurite length in neurons in (I). (K) Effect of Maf1 over-expression on Reelin-induced neurite outgrowth. Primary cortical neurons (3 DIV) were transfected with control vector or pCMV-Myc-Maf1 plasmid, followed by incubation in Mock or Reelin conditioned medium for 8 days, and total neurite length was measure as described in (F). (L) IF analysis of dendritic spine morphology. Primary hippocampal neurons transfected with scramble or Maf1 shRNA were incubated in Mock or Reelin conditioned medium for 8 days and then analyzed by IF using beta-tubulin III for spine morphology. (M) IF analysis of dendritic spine morphology. Primary hippocampal neurons transfected with control vector or pCMV-Myc-Maf1 plasmid were incubated in Reelin conditioned medium for 8 days and then analyzed by IF using beta-tubulin III for spine morphology. (N) A working model for Reelin-induced dendrite development.

REFERENCES

Beffert, U., Weeber, E., Durudas, A., Qiu, S., Masiulis, I., Sweatt, J., Li, W., Adelmann, G., Frotscher, M., Hammer, R., and Herz, J. (2005). Modulation of synaptic plasticity and memory by Reelin involves differential splicing of the lipoprotein receptor Apoer2. Neuron *47*, 567-579.

Boguta, M. (2012). Maf1, a general negative regulator of RNA polymerase III in yeast. Biochim Biophys Acta.

D'Arcangelo, G., Miao, G., Chen, S., Soares, H., Morgan, J., and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature *374*, 719-723.

Don, A., Tsang, C., Kazdoba, T., D'Arcangelo, G., Young, W., and Zheng, X. (2012). Targeting mTOR as a novel therapeutic strategy for traumatic CNS injuries. Drug Discov Today *17*, 861-868.

Fatemi, S. (2001). Reelin mutations in mouse and man: from reeler mouse to schizophrenia, mood disorders, autism and lissencephaly. Mol Psychiatry *6*, 129-133.

Geiduschek, E., and Kassavetis, G. (2006). Transcription: adjusting to adversity by regulating RNA polymerase. Curr Biol *16*, R849-851.

Goodfellow, S., Graham, E., Kantidakis, T., Marshall, L., Coppins, B., Oficjalska-Pham, D., Gerard, M., Lefebvre, O., and White, R. (2008). Regulation of RNA polymerase III transcription by Maf1 in mammalian cells. J Mol Biol *378*, 481-491.

Goodfellow, S., and White, R. (2007). Regulation of RNA polymerase III transcription during mammalian cell growth. Cell Cycle *6*, 2323-2326.

Herring, A., Donath, A., Steiner, K., Widera, M., Hamzehian, S., Kanakis, D., Kolble, K., ElAli, A., Hermann, D., Paulus, W., and Keyvani, K. (2012). Reelin depletion is an early phenomenon of Alzheimer's pathology. J Alzheimers Dis *30*, 963-979.
Jaworski, J., and Sheng, M. (2006). The growing role of mTOR in neuronal development and plasticity. Mol Neurobiol *34*, 205-219.
Jossin, Y., and Goffinet, A. M. (2007). Reelin Signals through Phosphatidylinositol 3-Kinase and Akt To Control Cortical Development and through mTor To Regulate Dendritic Growth. Molecular and Cellular Biology *27*, 7113-7124.
Jung, H., Yoon, B. C., and Holt, C. E. (2012). Axonal mRNA localization and local protein synthesis in nervous system assembly, maintenance and repair. Nat Rev Neurosci *13*, 308-324.
Kantidakis, T., Ramsbottom, B. A., Birch, J. L., Dowding, S. N., and White, R. J. (2010). mTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1. PNAS *107*, 11823-11828.
Lee, G., Kim, S., Homayouni, R., and D'Arcangelo, G. (2012). Dab2ip regulates neuronal migration and neurite outgrowth in the developing neocortex. PLoS One *7*, e46592.
Li, H., Tsang, C., Watkins, M., Bertram, P., and Zheng, X. (2006). Nutrient regulates Tor1 nuclear localization and association with rDNA promoter. Nature *442*, 1058-1061.
Michels, A. A., Robitaille, A. M., Buczynski-Ruchonnet, D., Hodroj, W., Reina, J. H., Hall, M. N., and Hernandez, N. (2010). mTORC1 Directly Phosphorylates and Regulates Human MAF1. Mol Cell Biol *30*, 3749-3757.
Niu, S., Renfro, A., Quattrocchi, C., Sheldon, M., and D'Arcangelo, G. (2004). Reelin promotes hippocampal dendrite development through the VLDLR/ApoER2-Dab1 pathway. Neuron *41*, 71-84.
Niu, S., Yabut, O., and D'Arcangelo, G. (2008). The Reelin Signaling Pathway Promotes Dendritic Spine Development in Hippocampal Neurons. J Neurosci *28*, 10339-10348.
Nolan, T., Hands, R. E., and Bustin, S. A. (2006). Quantification of mRNA using real-time RT-PCR. Nat Protocols *1*, 1559-1582.
Shor, B., Wu, J., Shakey, Q., Toral-Barza, L., Shi, C., Follettie, M., and Yu, K. (2010). Requirement of the mTOR Kinase for the Regulation of Maf1 Phosphorylation and Control of RNA Polymerase III-dependent Transcription in Cancer Cells. J Biol Chem *285*, 15380-15392.
Swiech, L., Perycz, M., Malik, A., and Jaworski, J. (2008). Role of mTOR in physiology and pathology of the nervous system. Biochim Biophys Acta *1784*, 116-132.
Tsang, C., Liu, H., and Zheng, X. (2010). mTOR binds to the promoters of RNA polymerase I- and III-transcribed genes. Cell Cycle *9*, 953-957.
Tsang, C., Qi, H., Liu, L., and Zheng, X. (2007). Targeting mammalian target of rapamycin (mTOR) for health and diseases. Drug Discov Today *12*, 112-124.
Tsang, C., and Zheng, X. (2007). TOR-in(g) the nucleus. Cell Cycle *6*, 25-29.
Ventruti, A., Kazdoba, T., Niu, S., and D'Arcangelo, G. (2011). Reelin deficiency causes specific defects in the molecular composition of the synapses in the adult brain. Neuroscience *189*, 32-42.
Weeber, E. J., Beffert, U., Jones, C., Christian, J. M., Forster, E., Sweatt, J. D., and Herz, J. (2002). Reelin and ApoE Receptors Cooperate to Enhance Hippocampal Synaptic Plasticity and Learning. J Biol Chem *277*, 39944-39952.
Wei, Y., Tsang, C., and Zheng, X. (2009). Mechanisms of regulation of RNA polymerase III-dependent transcription by TORC1. EMBO J *28*, 2220-2230.
Willis, I., and Moir, R. (2007). Integration of nutritional and stress signaling pathways by Maf1. Trends Biochem Sci *32*, 51-53.

Supplemental information

Supplmental Experitmental Procedures

Reagents, constructs and antibodies

Cell culture medium and reagents were purchased from invitrogen. Pharmacological inhibitors were purchased from LC Laboratories. Recombinant Reelin was obtained as the conditioned medium of the stable cell line CER as previously described (Niu et al., 2004). Mock medium was prepared from parental 293-ENBA cell line as described (Ventruti et al., 2011). Maf1 specific 29mer shRNA constructs were expressed from retroviral GFP vector (pGFP-V-RS, OriGene). Vector overexpressing Myc-tagged Maf1 was constructed from pCMV6-AN-His-Myc vector (OriGene). Raptor shRNA was expressed from pLKO.1 vector (AddGene). The anti-bodies used in this study includes phospho-mTOR (Ser2448) (Cell Signaling Technology), phospho-4EBP1 (Thr37/46) (Cell Signaling Technology), phosphor-S6K (Thr 389) (Cell Signaling Technology), Raptor (Cell Signaling Technology), β-actin (Cell Signaling Technology), α-tubulin (Cell Signaling Technology), Maf1 (Abcam), β-tubulin III (Novus), anti-GFP (Thermo Scientific), mTOR (Tsang et al., 2010), anti-Myc (9E10) (Harlan Laboratories).

Animals handling

All animals used in this study were handled in accordance with a protocol approved by the Association for Assessment and Accreditation of Laboratory Animal Care committees at Rutgers Robert Wood Johnson Medical School and Rutgers, the State University of New Jersey. CD-1, timed pregnancy mice were obtained from Charles river. ICR mice were obtained from Taconic Farms.

Neuronal cell line, primary neuronal culture, treatments and transfection

Neuro2A cells were grown and maintained in DMEM (high glucose; Invitrogen) with 10% horse serum and 5% fetal bovine serum (Invitrogen) at 37°C in 5% CO_2. Induction of differentiation was carried out by incubation of cells in DMEM with 1% FBS for 12 hours. For primary cortical and hippocampal neuronal cultures, cerebral cortices and hippocampus were dissected from E15.5 mouse embryos. Neurons were dissociated using a Papain Dissociation Kit (BioWorthington). The dissociated primary neurons were cultured as previously described (Lee et al., 2012). Treatments of neurons with the indicated pharmacological inhibitors and/or Reelin or Mock conditioned medium were followed as described (Ventruti et al., 2011). To test knockdown efficiency by Western blot analysis, approximately 5×10^6 dissociated cells were transfected with shRNA constructs using an Amaxa nucleofector kit, and plated in a 6-well plate coated with poly-L-lysine.

Protein extraction and western blot analysis

Cells were lysed in RIPA buffer containing 50 mM Tris-HCL, pH 7.2, 150 mM NaCl, 1% NP-40, 2 mM EDTA, 0.1% SDS, 0.1 mM PMSF, Protease and phosphatase inhibitor cocktails (Roche). Samples (5-30 µg/lane) were analyzed on 8-12% SDS-PAGE.

Chromatin Immunoprecipitation (ChIP)

ChIP assay, sequences of primers used for PCR and PCR conditions were performed as described previously (Tsang et al., 2010).

Indirect Immunofluorescence and analysis of dendritic development

For immunofluorescence analysis of neurons, cerebral cortices were dissected from E15.5 embryos, and the isolated neurons were cultured for 2-3 DIV. Approximately 0.2×10^6 cells were

placed on a glass coverslip in a 24-well plate coated with poly-L-lysine. Cells were fixed with 4% formaldehyde solution (in PBS) for 15 min, rinsed with PBS, incubated in a blocking solution and incubated with primary and secondary antibodies as described (Niu et al., 2004). DNA was stained for 15 min with 50 ng/ml DAPI. Microscopic analysis was performed with an Olympus fluorescence microscope equipped with a digital camera. Quantitative and statistical analyses were performed as described (Lee et al., 2012).

Preparation of brain sections and Fluorescence analysis

Postnatal day 0 (P0) brains were dissected, fixed overnight in 4% paraformaldehyde (PFA) at 4ºC and then placed in 30% sucrose/PBS mix. Brains were frozen in OCT (Tissue-Tek) and sectioned coronally at 30 µm. The sections were collected in PBS. The free-floating brain sections were permeabilized with 0.1% Triton X-100/PBS for 10 minutes and incubated with 5% normal goat serum (invitrogen)/0.1% Triton X-100/PBS at room temperature for 1 hour. The sections were incubated with primary antibody at 4ºC for overnight and then with secondary antibody and Red dot2 at room temperature for 1 hour. After several washes with PBS, the tissue sections were mounted and imaged by confocal microscopy using a Yokogawa CSU-10 spinning disk attached to an inverted fluorescence microscope (Olympus IX50). A primary antibody was rabbit anti-Maf1 (Abcam, 1:200) and a secondary antibody was conjugated to AlexaFluor 488 (Invitrogen). Reddot 2 (Biotium) was used for nucleus counterstaining.

Supplemental References

Niu, S., Renfro, A., Quattrocchi, C., Sheldon, M., and D'Arcangelo, G. (2004). Reelin promotes hippocampal dendrite development through the VLDLR/ApoER2-Dab1 pathway. Neuron *41*, 71-84.

Tsang, C., Liu, H., and Zheng, X. (2010). mTOR binds to the promoters of RNA polymerase I- and III-transcribed genes. Cell Cycle *9*, 953-957.

Ventruti, A., Kazdoba, T., Niu, S., and D'Arcangelo, G. (2011). Reelin deficiency causes specific defects in the molecular composition of the synapses in the adult brain. Neuroscience *189*, 32-42.

Supplemental figure legends

Figure S1. mTOR regulates transcription of 5S rRNA and tRNA^{LEU} genes in differentiated Neuro2A cells. (A) ChIP analysis of mTOR in differentiated Neuro2A cells in the absence or presence of rapamycin. PCR was used to detect the binding of mTOR to the promoters of 5S rRNA and tRNA^{LEU}. GAPDH was used as a negative control. (B) RT-PCR analysis of gene expression in differentiated Neuro2A cells treated with or without rapamycin (100 nM) for 3 hours. No DNA template (no DNA) or no reverse transcriptase (No RT) were served as controls. The primer pairs were used for detecting the nascent transcripts of precursor 5S rRNA and tRNALEU, which are processed rapidly during transcription and therefore can accurately reflect their transcription rates. Beta actin was used as a laoding control. Lower panel: Quantification of RT-PCR results. Data are presented as mean ± standard deviation (n=3). Asterisks, $p < 0.01$ for rapamycin untreated versus rapamycin-treated samples.

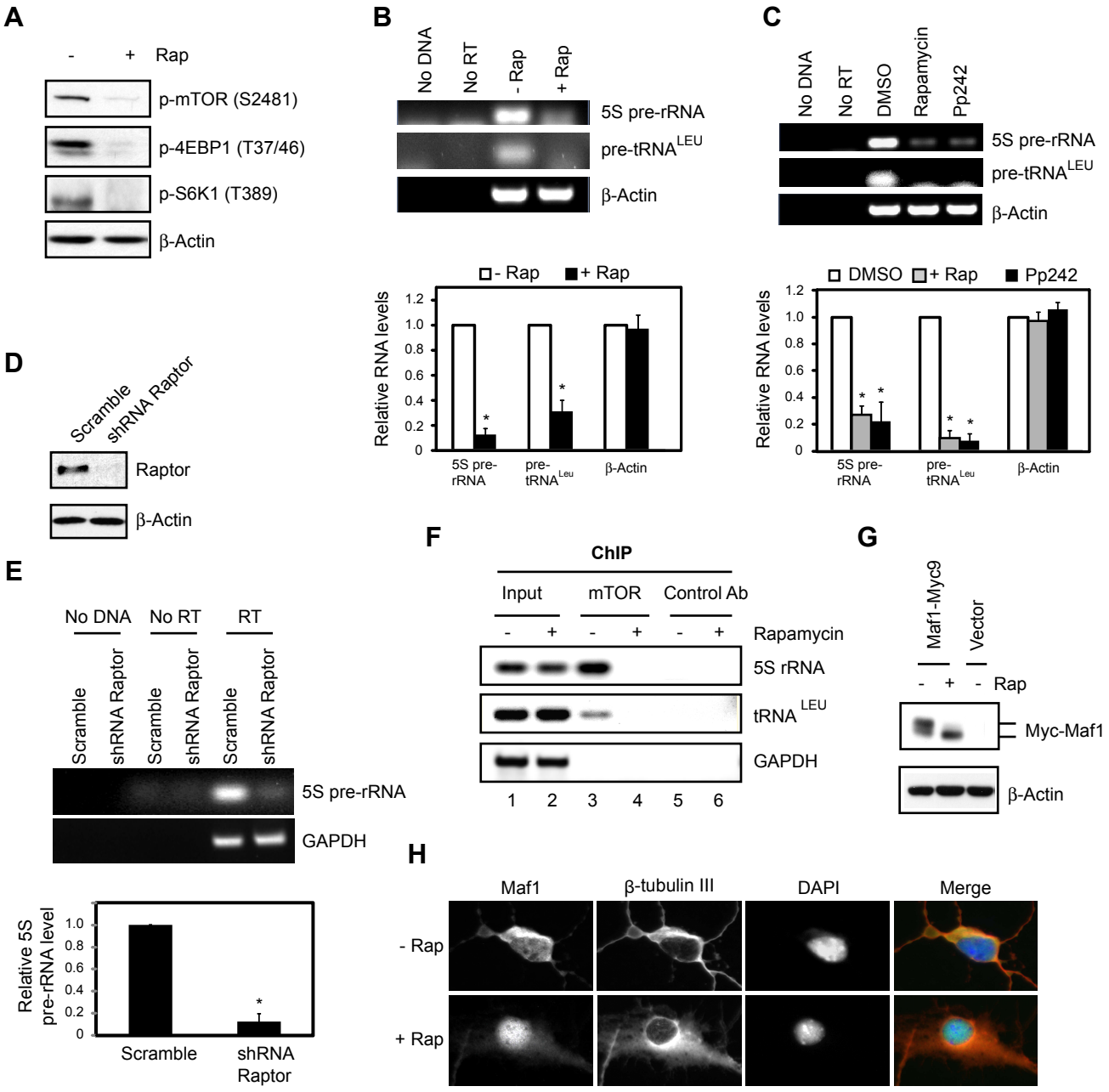
Figure S2. mTOR inhibition by rapamycin causes Maf1 accumulation in the nucleus in primary cortical neurons. Indirect immunofluorescence (IF) analysis of Maf1 localization in primary cortical neurons. The primary neurons of 5 div were treated with or without rapamycin and localization of Maf1 was analyzed by anti-Maf1 antibody. Cell and neurite morphology were analyzed by beta-tubulin III staining and nucleus was stained by DAPI.

Figure S3. Signaling characterization of Reelin-induced transcription regulation in primary hippocampal neurons. (A) RT-PCR analysis of 5S pre-rRNA in the extracts from primary hippocampal neurons of 3 DIV pre-treated with rapamycin or Ly294002 for 30 min, followed by incubation in Reelin conditioned medium containing the same concentration of drug for 20 min. (B) Quantification of result of (A). (C) RT-qPCR analysis of tRNA^{LEU} in RNA extract from (A).

Figure S4. Rapamycin inhibites and Reelin stimulates 5S rRNA transcription in primary hippocampal neurons.

RT-PCR analysis of 5S pre-rRNA in the extracts of primary hippocampal neurons treated with or without rapamycin, or incubated in Mock or Reelin conditioned medium. (C,D) RT-qPCR analysis of tRNA TYR and tRNALEU in the extracts of primary hippocampal neurons incubated in Mock or Reelin conditioned medium.

Figure 1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+ Rap
5S pre-rRNA
pre-tRNA LEU
β-Actin
- Rap + Rap
Relative RNA levels
5S pre-rRNA
pre-tRNA Leu
β-Actin
C
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Raptor
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Relative 5S pre-rRNA level
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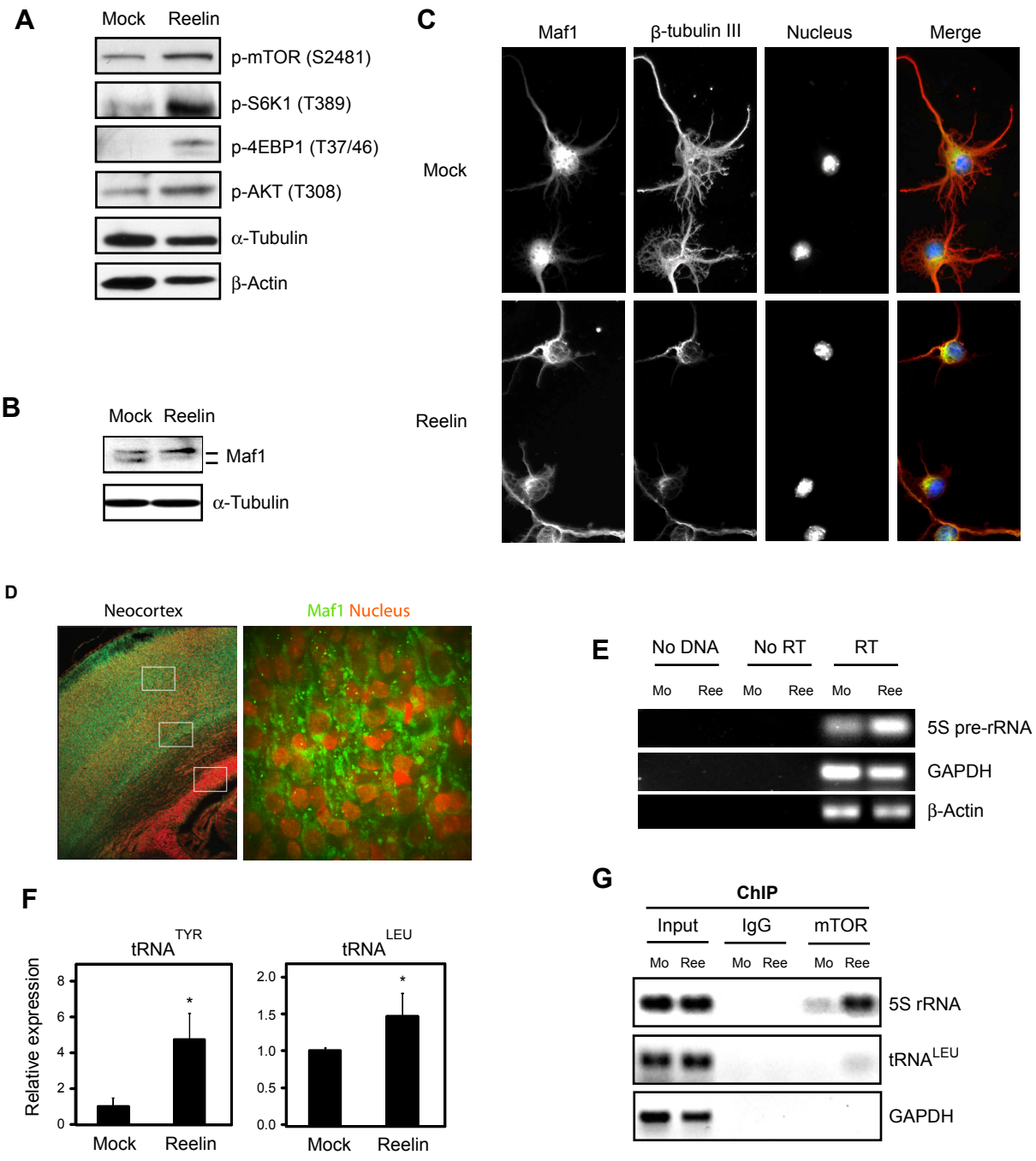
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β-Actin
B
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Reelin
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C
Maf1
β-tubulin III
Nucleus
Merge
Mock
Reelin
D
Neocortex
Maf1 Nucleus
E
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No RT
RT
Mo
Ree
Mo
Ree
Mo
Ree
5S pre-rRNA
GAPDH
β-Actin
F
tRNA TYR
tRNA LEU
Relative expression
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Reelin
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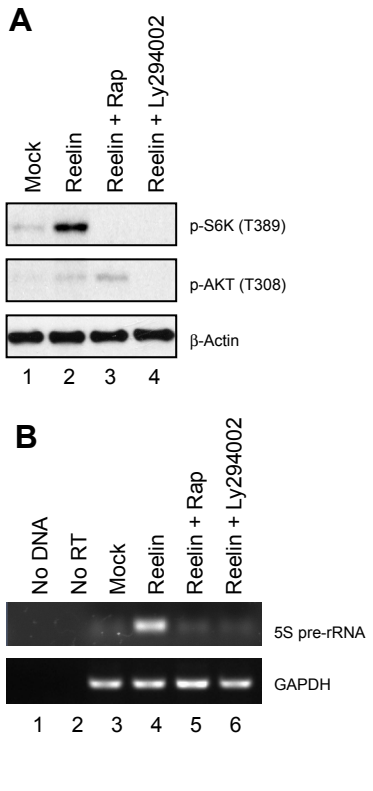
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Reelin
Reelin + Rap
Reelin + Ly294002
5S pre-rRNA
GAPDH
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C
Relative 5S pre-rRNA level
5S pre-rRNA
Mock
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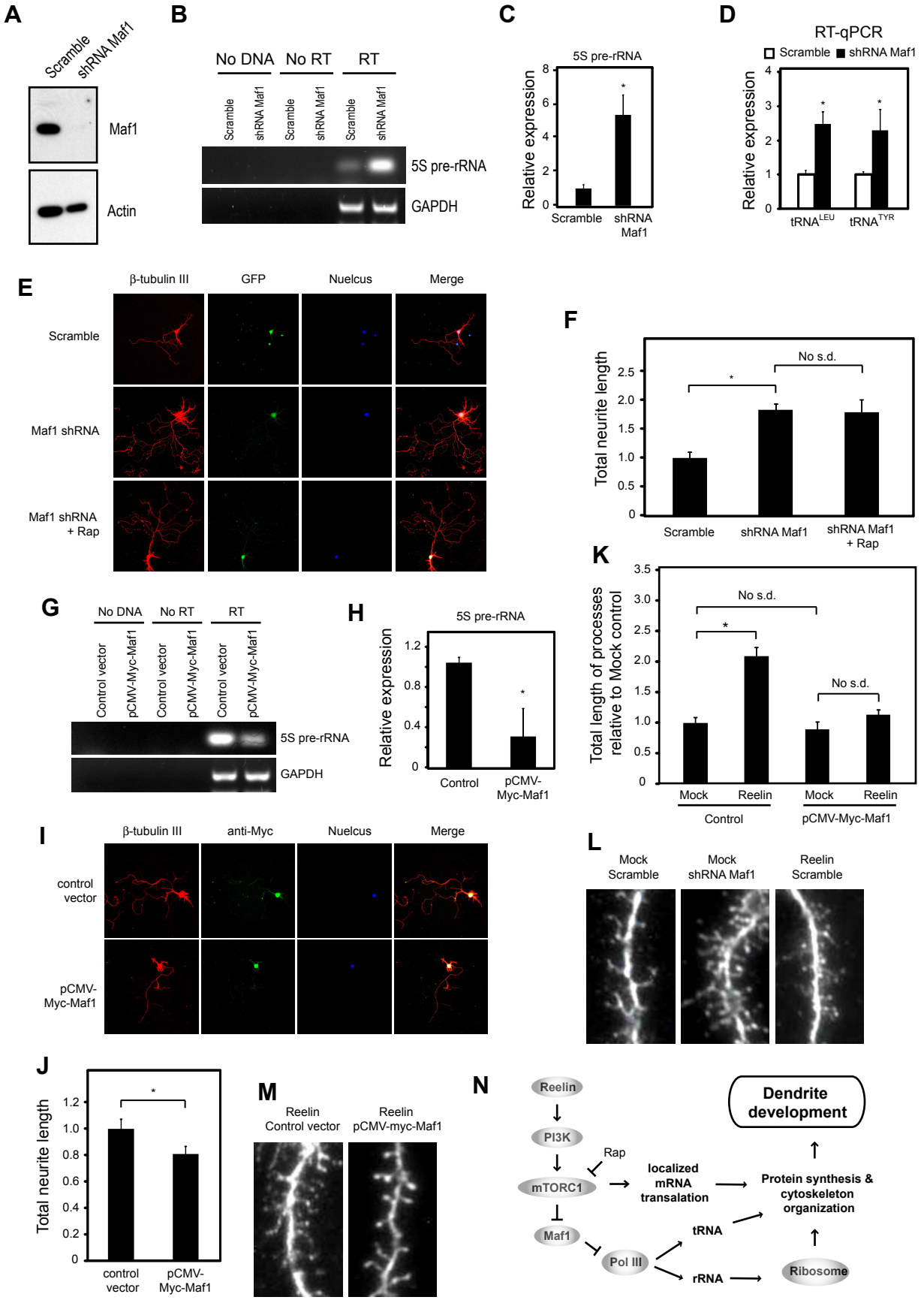
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Relative expression
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D
RT-qPCR
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shRNA Maf1
Relative expression
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GFP
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rRNA
Protein synthesis & cytoskeleton organization
Ribosome
Dendrite development